Results. Thapsigargin treatment results in the transient elevation of cytosolic calcium levels and the depletion of ER calcium stores (Hofer and Machen, Proc. Nat. Acad. Sci. 90:2598-2602 (1993), Montero *et al.*, J. Cell Biol. 139:601-611 (1997)). While this activity underlies the proposed therapeutic benefit of these compounds in CF, it is possible that it may also produce toxic side effects by activating calcium-dependent processes in a wide variety of cells (Berridge, Mol. Cell. Endocrin. 98:119-24 (1994)). Since the primary affected organ in CF is the lung (Davis *et al.*, Am. J. Respir. Crit. Care Med. 154:1229-1256 (1996); Pilewski and Frizell, Physiol. Rev. 79:Suppl: S215-S255 (1999); Rosenstein and Zeitlin, Lancet 351:277-282 (1998); Johnson *et al.* Nature Gen. 2:21-25 (1992)), correction of the CF defect in airway epithelial cells would dramatically reduce the morbidity associated with this disease. It is important, therefore, to determine whether therapeutically efficacious doses of thapsigargin applied directly to the lung by inhalation are clinically tolerable.

To examine this issue, six mice were exposed for 3 to 4 hours per day for 14 days to a nebulized solution of 1 μM thapsigargin in normal saline. The animals exhibited no obvious ill effects either during or between treatments. At the end of the 2 week trial, the animals were sacrificed and 4 were processed for histopathologic examination of the lungs. In all cases, the cellular architecture of the lungs (*i.e.*, alveolar and bronchiolar architecture) was completely normal (See Figure 8). One of the specimens exhibited a moderate peribronchiolar lymphocytic infiltration, while in the other 3 the density of peribronchiolar lymphocytes was within normal limits (data not shown).

To ensure that the dose of thapsigargin received by the mice was sufficient to rescue ΔF508-CFTR in airway epithelial cells, we examined the effect of nebulized thapsigargin on ΣCFBE290⁻cells. These airway epithelial cells were cultured on permeable filter supports and grown with an air-liquid interface. Thus, their apical membranes are separated from the atmosphere by only a thin film of fluid, as are the apical membranes of airway epithelial cells *in situ* (Davis *et al.*, Am. J. Respir. Crit. Care Med. 154:1229-1256 (1996); Pilewski and Frizell, Physiol. Rev. 79:Suppl: S215-S255 (1999)). Filter-grown ΣCFBE290⁻ cells were exposed to 1 μM nebulized thapsigargin for 3 hours and the distribution of ΔF508-CFTR was evaluated by immunofluorescence.

As can be seen in Figure 5, treatment of cells with nebulized thapsigargin was sufficient to produce a dramatic redistribution of ΔF508-CFTR to the apical

plasmalemma. Since the upper airway epithelial cells in the mice must have experienced a dose of thapsigargin similar to that received by the cultured cells, it would appear that mice tolerate long-term doses of thapsigargin sufficient to produce a clinical effect without experiencing any readily detectable or significant physiologic morbidity.

Experiment 5. Secretion of α1-antitrypsin from secretion incompetent null variant affected-hepatocytes after thapsigargin treatment.

Experiment 2 is repeated using a cell line that expresses a retention mutation for α1-antitrypsin, such as the secretion-incompetent variant, null (Hong Kong), retained in stably transfected mouse hepatoma cells (J. Biol. Chem. 269:7514-7519 (1994)). Changes in the cell phenotype are assessed by assaying cells for secretion of α1 antitrypsin (detailed description in J. Biol. Chem. 268:2001-2008 (1993)).

Briefly, cell monolayers are pulse labeled with [^{35}S] methionine for 30 minutes, after which the radiolabeled media is removed and replaced with media containing an excess of unlabeled methionine. During the chase period, one set of monolayers is treated with 1μM thapsigargin for 3 hours, while another set is incubated for 3 hours in drug free media. Secretion of α1 antitrypsin into the media is assessed by immunoprecipitation followed by electrophoresis and autoradiography.

Results. Cells expressing the secretion-incompetent variant of α1-antitrypsin, null (Hong Kong), are pulse labeled for 30 minutes with [^{35}S] methionine, after which they were incubated in non-radioactive media for 3 hours in the presence or absence of 1 μM thapsigargin. After this chase incubation, the media is collected and subjected to immunoprecipitation with anti α1 antitrypsin antibodies. Immunoprecipitates are analyzed by SDS-PAGE followed by autoradiography.

Radiolabeled α1-antitrypsin protein is present in the media from thapsigargin treated cells and is absent from media collected from untreated cells. These results demonstrate that thapsigargin treatment releases the mis-folded α1 antitrypsin protein from the endoplasmic reticulum and allows it to be secreted from the cell.

Experiment 6. Toxicity Tests for Thapsigargin.

Genetically uniform lab mice were given either normal drinking water (control) or drinking water which contained thapsigargin (1μM final concentration). The non-control group of mice were given the thapsigargin-treated water over a 3 to 7 day time period. There were no deaths, illnesses or side effects noted in the mice that were given the thapsigargin water (same as control group).

Experiment 7. Western Blot Analysis Establishes Maturation of the ΔF508-CFTR Protein in Thapsigargin-Treated Cells.

Materials and Methods. CFPAC cells were grown to confluence in 10 cm2 plates (Corning Costar, Cambridge, MA). Following thapsigargin treatment performed as described in Example 3, cells were harvested by scraping in PBS, lysed by sonication, and a crude membrane pellet was recovered by centrifugation at 50,000 x g for 2 hrs. Electrophoresis and Western blotting were performed as described (Gottardi, C.J. and Caplan, M.J., *J. Cell. Biol.* 121, 283 (1993)). CFTR protein was detected using an antibody directed against the CFTR nucleotide binding domain 1 (Catalog number 05585, clone L12B4) from Upstate Biotechnology (Lake Placid NY).

Results. Figure 6 presents a Western blot comparing the level of mature CFTR in thapsigargin treated and untreated CFPAC cells. Lane 3 is a positive control showing the ~170 kDa mature form of the ΔF508-CFTR protein in T84 cells. In untreated CPFAC cells no mature CFTR could be detected in whole lysates, consistent with the retention and degradation of the ΔF508-CFTR protein in the ER (lane 1). Lysates of thapsigargin-treated cells contained the ~170 kDa mature form of the ΔF508-CFTR protein, indicating that the protein had been released from the ER and allowed to proceed along the biosynthetic pathway through the Golgi complex (lane 2).

Experiment 8. Thapsigargin Treatment Can Induce Reversal of a Phenotypic Defect in CF Mice.

Materials and Methods. CF mice, which were the kind gift of Mitch Drumm, have had the ΔF508 mutation introduced into their endogenous copies of the *CFTR* gene by homologous recombination and are homozygous for the ΔF508 mutation. Construction of these mice is described in Zeiher, G.B., *et al.*, A mouse model for the deltaF508 allele of cystic fibrosis. *J. Clin. Invest.* 96: 2051-2064, 1995, and additional studies of these mice are described in Steagall WK and Drumm ML, Stimulation of cystic fibrosis transmembrane conductance regulator-dependent short-circuit currents across DeltaF508 murine intestines. *Gastroenterology*, 116(6):1379-88, 1999.

Nasal potential difference was measured essentially as described in Grubb, B.R., Vick, R. N., and Boucher, R.C., Hyperabsorption of Na^+ and raised Ca^{2+}-mediated Cl^- secretion in nasal epithelia of CF mice, *Am. J. Physiol.*,266: C1478-1483, 1994 and Ramjeesingh, M., *et al.*, Assessment of the efficacy of in vivo CFTR protein replacement therapy in CF mice, *Hum Gene Ther.*, 9(4):521-8, 1998.